Role of Plasmid-Borne Genes in the Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) by Consortium of Aerobic Heterotrophic Bacteria

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Abstract

**Background/Aim:** Certain plasmids play an important role in adaptation of natural microbial populations to oil and other hydrocarbons. This research investigated the role of plasmid-borne genes in the biodegradation of polycyclic aromatic hydrocarbons (PAHs) by a consortium of aerobic heterotrophic bacteria (AHB).

**Methods:** Physicochemical and microbiological characteristics of the water sample were analyzed using standard methods. AHB with the capacity for biodegradation were isolated from hydrocarbon polluted water from Bodo Creek using Bushnell Haas Agar. The positive isolates were subjected to plasmid profiling and curing. Three treatments: cured, uncured and control were used in the study. Curing was achieved with acridine orange. The degradation experiment was carried out in a 10 L glass bioreactor and monitored for 56 days using GC-MS analysis.

**Results:** Seven (7) out of 19 AHB isolates were selected for the degradation study. The 7 isolates, G1, G3, G19, GA5, GB3, GD1 and G12 were classified as *Shewanella halocit*is G1, *Shewanella* sp. G3, *Vibrio alginolyticus* G19, *Pseudomonas putida* G45, *Bacillus cereus* GB3, *B. pumilus* GD1 and *Shewanella* sp. G12, respectively based on the phylogenetic analysis of their 16S RNA genes. The sequences were deposited at the GenBank under the accession numbers KT886070-KT886076. The day 0 PAHs for cured, uncured and control treatments was 61.83 mg/L. After 56 days, the PAHs decreased to 2.90, 1.87 and 57.65 mg/L for cured, uncured and control treatments, respectively; representing percentage degradation of 95.31, 96.98 and 6.76%. The respective derived PAH degradation models for cured, uncured and control treatments were $32.614e^{-0.047t}$, $30.09e^{-0.05t}$ and $-0.0769t + 61.656$. There was no significant difference (p<0.05) between PAH degradation in cured and uncured treatments.

**Conclusion:** The study demonstrated that PAH degradation by the consortium used in the study was chromosomal and not plasmid-borne.

Keywords: Aerobic heterotrophic bacteria; Polycyclic aromatic hydrocarbons; Plasmid; Curing; Gas chromatography-mass spectrometry

**Introduction**

Petroleum-based products are the major source of energy for industry and daily life. Increase in its exploration and production has brought about high rate of environmental pollution involving both terrestrial and aquatic habitat [1]. The release of hydrocarbons into the environment whether accidentally or due to human activities often pose severe, immediate and long-term ecological and environmental repercussion, since a lot of hydrocarbon constituents are toxic and persistent in terrestrial and aquatic environments. Several physicochemical methods of decontaminating the environment have been established and employed. However, such methods are usually established and labour-intensive and often involve the risk of spreading the pollution because the waste would require disposal elsewhere. A better way would be to use biodegradation [2] (Figure 1).

Ogoniland in the Niger Delta region of Nigeria where the research was carried out has witnessed an intermittent discharge of crude oil into its environment since the inception of crude oil exploration and exploitation. This trend has led to the destruction of its farmlands, aquaculture, rivers and creeks with hydrocarbon compounds [3].

PAHs are formed naturally during thermal geological reactions associated with fossil-fuel and mineral productions and during burning of vegetation in forest and bush fires [4,5]. Anthropogenic sources, particularly fuel combustion, automobiles, spillage of petroleum products, and waste incinerators are significant sources of PAHs into the environment. Aromatic hydrocarbons are among the most prevalent and persistent pollutants in the environment.

Certain plasmids play an important role in adaptation of natural microbial populations to oil and other hydrocarbons. Some of the microbial catabolic pathways responsible for the degradation, including the *alk* (C, to C₅, n-alkanes), *nah* (naphthalene) and *xyl* (toluene) pathways have been extensively characterized and are generally located on large catabolic plasmids [6]. Furthermore, many reports have described and characterized microorganisms that can catabolize both aliphatic and aromatic hydrocarbons [7]. Several environmental isolates of *Acinetobacter* sp. and *Alcaligenes* sp. *Arthrobacter* sp. and two *Rhodococcus* strains have been found to degrade both alkanes and naphthalene, although the genes and catabolic pathways responsible were not described [8-10].

Plasmids are also important in the development (transformation) of new organisms with enhanced degradative capability. Many bacteria of diverse genera have extra chromosomal DNA, suggesting the widespread presence of plasmids in prokaryotic cells. Thus the ability

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to identify plasmids in bacterial systems is important. Using molecular biology techniques, it is possible to slice pieces of DNA containing genes for specific degradative pathways into plasmids. These plasmids can then be introduced into a host organism resulting in a recombinant or genetically engineered microorganisms (GEM) with new degradative capabilities. These are used to remediate contaminated sites mainly as organisms for bioaugmentation [11]. Appropriate environmental factors are however, essential for the performance of these organisms [12].

This study was aimed at evaluating the role of plasmid-borne genes in the biodegradation of polycyclic aromatic hydrocarbons (PAHs) by a consortium of aerobic heterotrophic bacteria isolated from Ogoniland in the Niger Delta area of Nigeria.

Materials and Methods

Site description

The aerobic heterotrophic bacteria used in this study were isolated from Bodo Creek brackish water contaminated with crude oil. Bodo Creek is located in Bodo community of Gokana Local Government Area, in the Niger Delta Basin of Nigeria.

Sample collection

Five (5) samples of hydrocarbon polluted brackish surface water from a distance of 50 m apart at 5 points were collected with a sterile container and taken to environmental microbiology laboratory of the University of Port Harcourt for the isolation of hydrocarbon utilizing bacteria.

Physicochemical analysis

The baseline physicochemical characteristics of the water samples were analyzed. The parameters that were analyzed using standard methods as described by APHA included: nitrate, total nitrogen, phosphate, total organic carbon, pH, temperature, electrical conductivity, sulphate and turbidity. Salinity was determined using refractometer (S/mill-E Range 0-100%) [13]. The Gas chromatographic analyses were carried out to estimate the polycyclic aromatic hydrocarbons (PAH) and the total petroleum hydrocarbons (TPH). TPH was analyzed using the USEPA 8015. Sample extraction was carried out with dichloromethane followed by sample clean-up and separation. One microliter (1 µl) of extract after concentration to 1ml was injected into the Agilent 6890 GC-FID for analysis. The concentration of TPH was electronically deduced from the calibration graph.

Polycyclic aromatic hydrocarbons (PAH) were analyzed using the USEPA 8270. Sample extraction was carried out with dichloromethane followed by sample clean-up and separation. One microliter (1 µl) of extract after concentration to 1ml was injected into Agilent 7890 GC-MS for analysis. The concentration of the individual component of PAH was automatically deduced from the calibration graph (Figure 2).

Microbiological analysis

Serial dilution of sample: Ten-fold serial dilution was performed on the samples as described by Jalal [14].

Isolation and enumeration of hydrocarbon utilizing bacteria: Exactly 0.1 ml each of 10⁻³, 10⁻⁴ and 10⁻⁵ dilutions of the serially diluted samples were spread onto Bushnell Haas Agar medium (g/L: MgSO₄·7H₂O- 0.2g; CaCl₂·2H₂O- 0.02g; KH₂PO₄- 1g; K₂HPO₄- 1g; NH₄NO₃- 1g; FeCl₃- 0.05g; Agar 20g; nystatin- 0.5g; pH 7.0 ± 0.2) in duplicates. The medium was amended with 5% crude oil and sterilized in an autoclave at 121°C (15 psi) for 15 min). The plates were incubated at 37°C for 4 days. The plates with colonies ranging between 30 and 300 were selected and the average taken as the hydrocarbon utilizing bacterial count. Discrete colonies from the agar plates were selected based on their difference in morphology and sub-cultured on nutrient agar to obtain pure culture of the isolates. The pure cultures were maintained in minimal salt agar slant for further studies. The total viable count (TVC), expressed in colony forming units per ml (cfu/ml) was calculated using the formula below:

\[ \text{TVC (cfu/ml)} = \frac{\text{No of colonies} \times \text{dilution factor}}{\text{Volume of inoculum}} \]

Preliminary biodegradation test: The preliminary biodegradation test was carried out in test tubes containing Bushnell Haas broth amended with 1% crude oil. The isolates were inoculated into each test
Plasmid curing by treatment with acridine orange: The plasmid curing was performed using the method described by Silhavvy with slight modification. Bacterial cells were grown in broth overnight [17]. Five (5) ml of Nutrient broth supplemented with 0.1 mg/ml acridine orange was prepared. The organisms were sub-cultured into Nutrient broth containing the acridine orange, incubated at 37°C from 48 h to about one week and plated out on nutrient agar. Colonies able to grow on nutrient agar but on solid medium were isolated and considered cured. These colonies were subjected to plasmid profiling to confirm loss of plasmid.

Experimental design

The final experiment comprised three set-ups namely: cured set-up, uncured and control. In the cured set-up the consortium cured of plasmid were used to inoculate the crude oil contaminated water sample with crude oil concentration of 5% v/v. The uncured set-up contained the same crude oil concentration but with isolates still retaining their plasmids while the control set-up contained no inoculum.

Biodegradation experiment

The degradation experiment was carried out in a 25 L glass bioreactor and monitored for TPH, PAH, pH, and microbiological parameters (count of hydrocarbon utilizing bacteria and total heterotrophic bacteria) fortnightly within a period of 56 days (Figure 3).

Characterization of the selected bacterial consortium

Phenotypic characterization: The selected bacterial isolates were subjected to several biochemical methods as described by Holts, MacFaddin and Madigan [18-20].

Chromosomal DNA extraction: DNA extraction, PCR amplification of the bacterial 16S rRNA genes and gel electrophoresis of the isolate were carried out at the Molecular Biology Laboratory of National Institute for Medical Research (NIMR), Yaba, Lagos, Nigeria. The PCR product was sent to GATC Biotech AG (European Genome and Diagnostics Centre - Jakob-Stadler-Platz 7, 78467 Constance, Germany) where the Sanger Sequencing was carried out. DNA
results were compared by one-way analysis of variance (one-way ANOVA) and multiple range tests to find the differences between the measurement means at 5% (0.05) significance level using IBM SPSS Statistics Version 20.0 (Gailly and Adler, US).
Plasmid profiling and curing

Twelve (12) out of the nineteen (19) bacterial isolates showed the presence of degradative plasmid with size ranging between 2027 and 23130 bp.

Selection of consortium for biodegradation

The result of the preliminary biodegradation experiment showed that the twelve isolates were capable of degrading PAH hydrocarbon components however 7 isolates (G1, G3, G12, G19, A5, B3 and D1) which showed higher optical density values were selected for the proper biodegradation test.

PAH analysis of bioreactor treatments

The PAH components present in the Bonny light crude oil as detected by GC-MS analysis were naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benz(a)pyrene, dibenz(g,h,i)anthracene, indeno(1,2,3-c,d)pyrene and benz(g,h,i)perylene for both cured and uncured treatment on day 0. The total amount of PAH was 61.83 mg/L. The total amount of PAH after contamination of water with crude oil and inoculums showed that cured, uncured and control had PAH of 61.83 mg/L respectively (Table 2). PAH values during the experiment showed reduction for the cured treatment from 61.83 mg/L to 2.90 mg/L, uncured from 61.83 mg/L to 1.87 mg/L and control from 61.83 mg/L to 57.65 mg/L for day 0 and day 56 respectively. The percentage degradation of PAH was 96.98, 95.31, and 6.76% for the uncured, cured and control treatments, respectively in 56 days.

PAH degradation modeling in cured, uncured and control treatments

PAH degradation was monitored for a period of eight (8) weeks (56 days) for the uncured, cured and control treatments. Mathematical modeling of PAH degradation through non-linear regression method revealed that PAH content degradation followed an exponential pattern for the uncured and cured samples while that of the control followed a linear curve. The respective derived PAH degradation models for uncured, cured and control treatments are 30.09e-0.05t, 32.614e-0.047t and -0.0769t + 61.656. The R² values for the uncured, cured and control models are 0.7939 (79%), 0.8051 (81%) and 0.9707 (97%) respectively.

PCR amplification of 16S rRNA gene

All the 7 isolates showed amplification with an amplicon size of 500 bp.

Phylogenetic tree analysis of the isolates

Phylogenetic tree analysis of the sequences generated from the isolates revealed that the isolates belonged to four genera namely: *Bacillus*, *Pseudomonas*, *Shewanella* and *Vibrio*. The phylogenetic tree constructions are given in Figure 6. The 7 isolates, G1, G3, G19, GA5, GB3, GD1 and G12 were classified as *Shewanella haliotis* G1, *Shewanella sp*. G3, *Vibrio alginolyticus* G19, *Pseudomonas putida* GA5, *Bacillus cereus* GB3, *B. pumilus* GD1 and *Shewanella sp*. G12.

Discussion

In this study, 19 hydrocarbon utilizing bacteria were isolated from Bodo Creek brackish water and screened for hydrocarbon degradation as well as the presence of plasmids. Out of these 19 isolates, 7 isolates (G1, G3, G19, GA5, GB3, GD1 and G12, classified as *Shewanella haliotis* G1, *Shewanella sp*. G3, *Vibrio alginolyticus* G19, *Pseudomonas putida* GA5, *Bacillus cereus* GB3, *B. pumilus* GD1 and *Shewanella sp*. G12, respectively based on the phylogenetic analysis of their 16S rRNA genes) were chosen for the biodegradation study based on their first, hydrocarbon utilizing capabilities and then the presence of degradative plasmids.

Many researchers (Onifade and Abubakar, Chikere and Ekwuabu, Ichor) have reported the isolation of hydrocarbon utilizing bacteria from contaminated brackish water [24-26]. The bacterial strains used in this study showed poly cyclic aromatic hydrocarbon degradation ability. Said reported the degradation of PAH by *Shewanella* species

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Uncured (mg/L)</th>
<th>Cured (mg/L)</th>
<th>Control (mg/L)</th>
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<tr>
<td>0</td>
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<td>56</td>
<td>1.87</td>
<td>2.90</td>
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Percentage degradation (%)

96.98, 95.31, 6.76

Table 2: Summary of PAH content of the treatments during the biodegradation experiment.
[27]. Other researchers Head, Pucci have reported the involvement of *Shewanella* species in the degradation of crude oil [28,29].

Hydrocarbon degradation by *Bacillus* species is well documented (Okpokwasili and Nnorom, Odokuma and Dickson, Okerentugba and Ezenyore, Ibine, Chikere and Ekwuabu, Ichor) [30-33]. Borah and Yadav [34] reported hydrocarbon degradation by *Bacillus cereus* strains. The result of this investigation is consistent with the above report as the *Bacillus cereus* IAM 12605 used in the study was able to degrade both the PAH components of the crude oil. Patowary reported biodegradation of polyaromatic hydrocarbons employing biosurfactant-producing *Bacillus pumilus* KS2 [35].

There are supports on the degradation of petroleum hydrocarbon by *Pseudomonas putida* strains. Ichor Vinothini have implicated *Pseudomonas putida* in the biodegradation of petroleum hydrocarbon. Their claim is consistent with the result obtained from this study. The isolated *Pseudomonas putida* strain LB22 was able to degrade PAH [36].

The bacterial isolates used in this study were found to all have plasmids. Plasmid carriage may provide genetic control for adherence to, or emulsification of, hydrocarbons, ultimately resulting in removal via biodegradative pathway(s) Okpokwasili [37]. However, there is dearth of information on the isolation of degradative plasmids in *Shewanella* spp. Many researchers have isolated and identified degradative plasmids in *Pseudomonas putida*. Worsley, Franklin isolated TOL plasmid pWWO from *Shewanella putrefaciens* [38,39].

In both cured and uncured treatments the consortium was able to efficiently degrade the crude oil. Although uncured treatment had slightly more percentage of the crude oil degraded, 96.98% loss of the PAH against 95.31% loss of the PAH in the cured treatments. This means that the removal of plasmid from the consortium did not result in the loss of biodegradation potential but however led to slightly decreased degradation. This result is supported by the study carried out by Akpe who observed that loss of plasmids by *Pseudomonas putida* MARS14 did not lead to complete loss of their degradative abilities. It only resulted in reduction in their degradation potential [12].

However, many workers have also shown that plasmids carrying genes influencing degradation of hydrocarbons abound in bacterial populations. Because these bacteria are widely distributed in chronically
polluted estuarine environments, plasmid carriage may provide genetic control for adherence to, or emulsification of, hydrocarbons, ultimately resulting in removal via biodegradative pathway(s). The work by Okpokwasili showed that plasmid DNA was found to be associated with clarification of phenanthrene-degrading Flavobacterium species [37].

Conclusion
This study showed that the aerobic heterotrophic bacterial consortium used were able to degrade petroleum hydrocarbon with or without the presence of their degradative plasmid. Although the percentage degradation in the uncured was slightly higher over the 56-day period monitored, there was no significant difference observed in the rate of hydrocarbon degradation at the end of the monitored period. The consortium of aerobic heterotrophic bacteria used in the study were identified as Shewanella sp. BAB 3791, Shewanella algae MARS14, Shewanella sp. ECSMB56, Vibrio alginolyticus CSMCRI-1113, Pseudomonas putida LB22, Bacillus cereus NB4, and Bacillus pumilus BF17 using their 16S RNA sequence analysis. The model developed from the work can be effectively used in predicting PAH and TPH concentration over time.

References